



# EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND  
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1323

## ADAPTIVE MECHANISMS UNDERLYING MICROBIAL RESISTANCE TO DISINFECTANTS

Lisa S. Smith  
Vipin K. Rastogi

RESEARCH AND TECHNOLOGY DIRECTORATE

Lalena Wallace  
Defense Threat Reduction Agency

February 2016

Approved for public release; distribution is unlimited.



#### Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 h per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
1. REPORT DATE (DD-MM-YYYY) XX-02-2016		2. REPORT TYPE Final		3. DATES COVERED (From - To) Nov 2011– Sep 2014	
4. TITLE AND SUBTITLE Adaptive Mechanisms Underlying Microbial Resistance to Disinfectants				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Smith, Lisa S.; Rastogi, Vipin K. (ECBC); and Wallace, Lalena (DTRA)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Director, ECBC, ATTN: RDCB-DRB-D, APG, MD 21010-5424 Defense Threat Reduction Agency, 8725 John J. Kingman Road, MSC 6201, Fort Belvoir, VA 22060-6201				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-1323	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Environmental Quality Basic Research and Development, Armament Research, Development and Engineering Center, Picatinny Arsenal, NJ 07806-5000				10. SPONSOR/MONITOR'S ACRONYM(S) EQBRD	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: This project was performed to determine if clinical pathogens evolve and acquire resistance to Lysol, an all-purpose cleaner and disinfectant (U.S. Environmental Protection Agency regulation no. 777-89 [ready-to-use (RTU) 1:16 dilution]). A clinical surrogate, <i>Escherichia coli</i> , was used in these studies. <i>E. coli</i> cells were grown in the absence or presence of Lysol. The parent strain (PS) was sensitive to the presence of 1.6% RTU strength Lysol. LR50, a resistant strain that showed resistance to 50% of the RTU strength Lysol, was derived through progressive subculturing. (A 30-fold increase in resistance to Lysol illustrates genome plasticity and adaptation of bacterial cells.) LR50 was subcultured in Tryptic soy broth five times, and then its resistance phenotype was confirmed in the presence of 50% Lysol. Biochemical characterization revealed the presence or absence of specific polypeptides unique to LR50. Genomic sequencing was done, and some single nucleotide polymorphisms were observed to be unique to LR50. Finally, altered antibiotic resistance was determined for LR50. In a separate set of experiments, the adaptive resistance of <i>E.coli</i> cells to 17% Germ-X (GR17), a hand sanitizer, was also observed. GR17 PS, a resistant strain, which was capable of growing in the presence of GR17, was derived during this study.					
15. SUBJECT TERMS Tryptic soy broth (TSB) <i>Escherichia coli</i> RTU strength Lysol Single nucleotide polymorphism (SNP) LR50 50% Lysol Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	22	19b. TELEPHONE NUMBER (include area code) (410) 436-7545

Blank

## **PREFACE**

The work described in this report was funded by U.S. Army Environmental Quality Basic Research and Development (EQBRD). The work was started in November 2011 and completed in September 2014.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

## **Acknowledgments**

The authors acknowledge funding from EQBRD managed by U.S. Army Armament Research, Development and Engineering Center (Picatinny Arsenal, NJ). We also acknowledge Savannah Maggio (Oak Ridge Institute for Science and Education, Oak Ridge, TN) for laboratory preparatory work, Mike Krepps (Excet, Springfield, VA) for genomic analysis, and Nicole Rosenzweig (U.S. Army Edgewood Chemical Biological Center) for facilitating the genome sequencing.

Blank

## CONTENTS

1.	INTRODUCTION .....	1
2.	MATERIALS AND METHODS.....	2
2.1	Materials .....	2
2.1.1	Bacterial Strains Used.....	2
2.1.2	Disinfectants Used .....	2
2.1.3	Reagents Used.....	2
2.2	Methods.....	2
2.2.1	Growth of <i>E.coli</i> .....	2
2.2.2	Biochemical Tests.....	3
2.2.3	Confirmation of the Disinfectant-Resistant Phenotype .....	3
2.2.4	Antibiotic Testing .....	3
2.2.5	Genomic Sequencing .....	3
3.	RESULTS .....	3
3.1	Growth Curves .....	3
3.2	Biochemical Tests.....	5
3.3	Antibiotic Resistance .....	6
3.4	Genomic Sequencing .....	7
4.	DISCUSSION .....	7
5.	CONCLUSIONS.....	8
	LITERATURE CITED .....	9
	ACRONYMS AND ABBREVIATIONS .....	11

## FIGURES

1.	Growth curve of LR50 strain in TSB.....	4
2.	Growth curve of LR50 strain in 50% Lysol.....	4
3.	Growth curve of PS in TSB .....	5
4.	Protein gel with both LR strains in 50% Lysol, LR strain in TSB, and control strain in TSB .....	5
5.	Antibiotic-resistant profiles of LR50 and PS for 9 antibiotics .....	6
6.	Antibiotic-resistant profiles of LR50 and PS for 10 antibiotics .....	6



# ADAPTIVE MECHANISMS UNDERLYING MICROBIAL RESISTANCE TO DISINFECTANTS

## 1. INTRODUCTION

The emergence of a new resistance mechanism in the “superbug” and its spread across bacterial species illustrate microbial adaptability to sublethal exposures of antimicrobials. The increasing use of disinfectants in clinical and household settings has raised serious concerns for the development of resistance mechanisms in bacterial cells (McDonnell et al., 1999). The mechanisms underlying each of these two phenomena may be quite different. A clearer understanding of biochemical and genetic adaptation is expected to offer new insight into countermeasure development.

Penicillin was first used to treat bacterial infections in the 1940s. By the 1950s, a penicillin-resistant strain of *Staphylococcus aureus* became common. Methicillin was introduced in 1961 to treat infections with such cells. Within one year, Methicillin-resistant *S. aureus* (MRSA) strains were observed. MRSA strains are resistant to a host of antibiotics, including vancomycin. Bacterial cells evolved and developed resistance to antimicrobial use, and the short generation times and large population size of bacteria helped boost this evolution.

Disinfectants are commonly used in water treatment plants and for cleaning surfaces in medical treatment facilities. In addition, household use of disinfectants has increasingly grown as these products typically kill 99.999% of pathogens within 5–10 min. Chlorine and quaternary ammonium compounds (quats) are the common key ingredients in these disinfectants. The general functions of disinfectants include destruction of cell membranes, interference with key biochemical functions, blockage of nutrient uptake, and decontamination of waste products. The increasing use of disinfectants raises the prospect of improper use (i.e., sublethal concentrations of disinfectants result in the adaptation of bacterial populations, leading to derivation of resistant isolates).

Mechanisms for disinfectant resistance of bacterial cells include the secretions of gelatinous exopolysaccharide, which some species can use to form biofilms. The use of an efflux pump to selectively export the disinfectant is another possible mechanism for resistance. Some bacteria may alter their gene expression of novel transporter proteins for protection.

This study compared disinfectant-resistant populations of *Escheria coli* with control sets of *E.coli* for physiological, biochemical, and genetic differences in an attempt to understand resistance mechanisms. Bacterial cells with increased resistance to disinfectants were analyzed for their resistance to antibiotics to investigate a possible correlation between these characteristics.

## **2. MATERIALS AND METHODS**

### **2.1 Materials**

#### **2.1.1 Bacterial Strains Used**

We used *E. coli*, a clinical surrogate, for the disinfectant-resistant tests. The *E. coli* was obtained from the American Type Culture Collection ([ATCC] Manassas, VA [ATCC number 11775]). *E. coli* is a gram-negative, facultative anaerobic, and rod-shaped bacteria commonly found in warm-blooded animals.

#### **2.1.2 Disinfectants Used**

We used Lysol brand disinfectant, four-in-one all-purpose cleaner (U.S. Environmental Protection Agency registration number 777-89). This disinfectant consists of alcohols and quaternary ammonium chlorides. Lysol contains the following ingredients: alcohols C12–16, ethoxylated (2.5–10%), Alkyl (50% C14, 40% C12, 10% C16) dimethyl benzyl ammonium chlorides (1–2.5%), and ethanol (0.1–1%). Alkyl dimethyl benzyl ammonium chloride attaches to the bacteria and causes the cytoplasmic membrane to leak, which damages and then kills the bacterial cells. The manufacturer recommended a ready-to-use (RTU) concentration of 1:16 of Lysol/Tryptic soy broth (TSB), which was regarded as 100% effective.

#### **2.1.3 Reagents Used**

TSB was used as the diluent in all the experiments. The RTU Lysol was further diluted in the ratio of 1:10 in a working stock.

Tryptic soy agar was used for plating the *E. coli* cells in all of the experiments.

### **2.2 Methods**

#### **2.2.1 Growth of *E. coli***

*E. coli* was grown in TSB overnight, and a micro-titer plate assay was used to screen the parent strain (PS) of *E. coli* for sensitivity. Sensitivity percent was then determined.

*E. coli* was grown over a 6 month period in increasing concentrations of Lysol. Growth curves were conducted for the PS *E. coli* cells, the Lysol-resistant (LR) strain in TSB, and another strain that showed resistance to 50% of the RTU strength Lysol (LR50). Growth curves were completed to determine the doubling time of the bacteria.

### **2.2.2 Biochemical Tests**

Protein extraction was performed from the bacterial cells. SDS-PAGE\* gels were run to compare the proteins in the PS and LR strains. The two strains were also examined using microscopy to compare the cell types.

### **2.2.3 Confirmation of the Disinfectant-Resistant Phenotype**

The genetic basis of the disinfectant phenotype of the LR strain was confirmed. The LR50 cultures were grown in TSB and subcultured five times. They were then grown in the presence of 50% Lysol.

### **2.2.4 Antibiotic Testing**

Nineteen antibiotics were tested against PS and LR50 strains, respectively. The following antimicrobial susceptibility test discs were tested: 15 µg of azithromycin, 15 µg of clarithromycin, 5 µg of rifampin, 5 µg of tetracycline, 2 µg of ampicillin, 10 µg of ampicillin, 2 IU of penicillin, 10 IU of penicillin, 30 µg of tetracycline, 5 µg of cefdinir, 30 µg of cefaclor, 5 µg of novobiocin, 10 µg of streptomycin, 10 µg of gentamicin, 20 µg of amoxicillin, 5 µg of ciproflaxin, 10 µg of bacitracin, 5 µg of vancomycin, and 100 µg of carbenicillin. The appearance of a zone of inhibition was proportional to bacterial resistance. Therefore, the zone of inhibition was measured in side-by-side tests of LR50 and PS samples. The zone of inhibition on the growth of the LR50 strain was caused by each antibiotic and compared with that of the growth on the PS test discs.

### **2.2.5 Genomic Sequencing**

The samples were prepped for genome sequencing using a Nextera (Illumina, Inc., San Diego, CA) sequencing kit and sequenced on an Illumina HiSeq 2000 sequencing system. A 2 × 100 run configuration was used (i.e., reads were produced in pairs, and each pair was 100 bases long). Each sample produced >5 gigabases. Data were analyzed using Bowtie sequencing analysis and SAMtools and CLC Bio genomics workbenches (CLC Bio, Qiagen, Aarhus, Denmark). DNA was isolated from the PS and LR50 strains. The genome sequences of both strains were analyzed and compared.

## **3. RESULTS**

### **3.1 Growth Curves**

Figures 1–3 show the growth curves and doubling times of the PS and LR50 strains in different cultures.

---

\*SDS-PAGE is sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis.

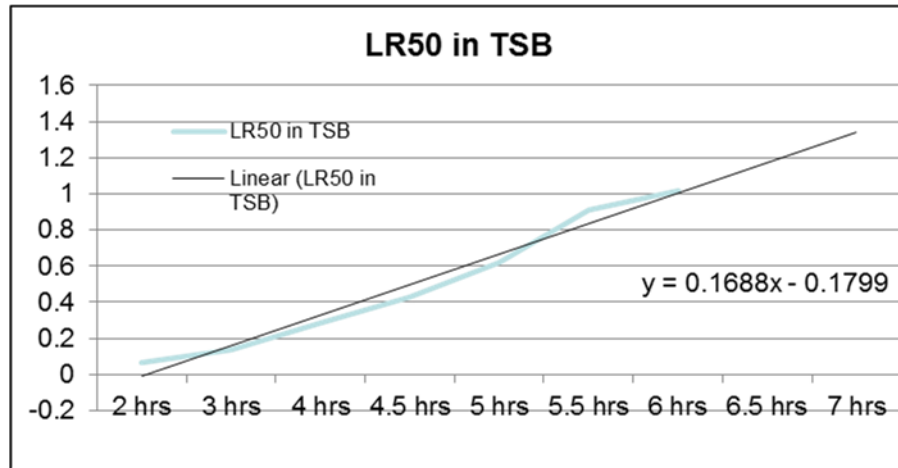


Figure 1. Growth curve of LR50 strain in TSB.  
The doubling time is 60 min.

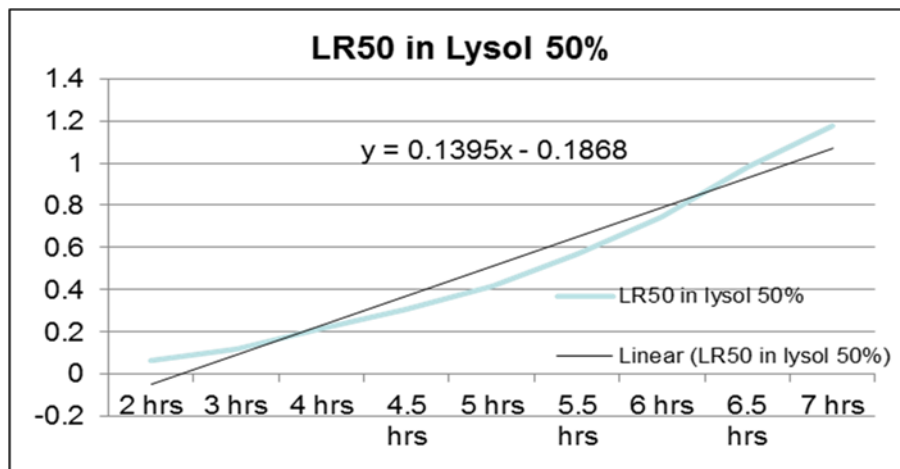


Figure 2. Growth curve of LR50 strain in 50% Lysol.  
The doubling time is 65 min.

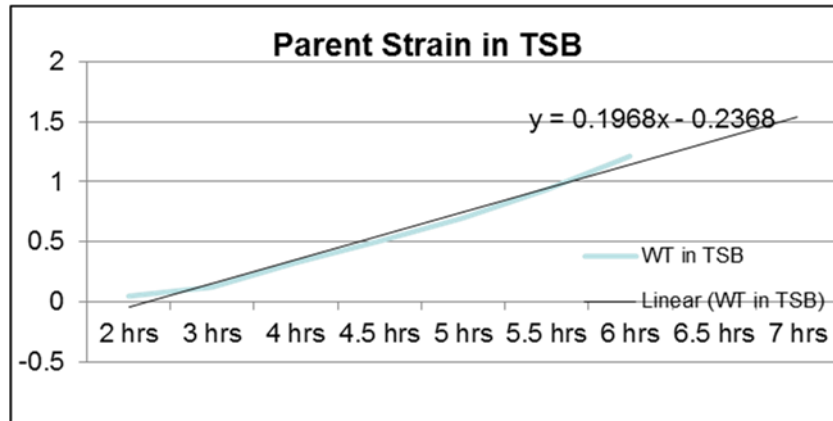


Figure 3. Growth curve of PS in TSB.  
The doubling time is 60 min.

Figure 4 shows that the LR strain grown in the 50% Lysol is missing a polypeptide of ~130 kDa, which is present in the LR and control strains grown in TSB. A polypeptide of ~100 kDa is uniquely present in the LR50 strain after it was grown in Lysol. Alterations in gene expression appeared to be the basis for the LR50 phenotype.

### 3.2 Biochemical Tests

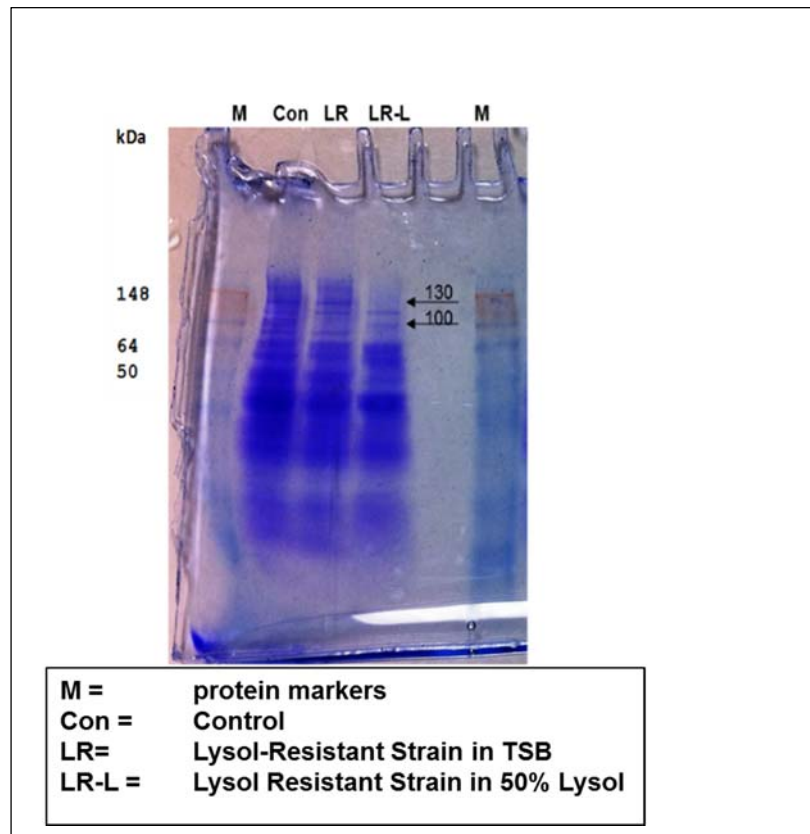


Figure 4. Protein gel with both LR strains in 50% Lysol, LR strain in TSB, and control strain in TSB.

### 3.3 Antibiotic Resistance

LR50 was resistant to two antibiotics, whereas the PS was not. LR50 had no zone of inhibition around 5 µg of rifampin and 10 IU of penicillin. On the other hand, the PS showed a zone of inhibition around these two antibiotics. Therefore, only LR50 was observed to be resistant to these two antibiotics.

Figures 5 and 6 show the antibiotic-resistant profiles of LR50 and PS.

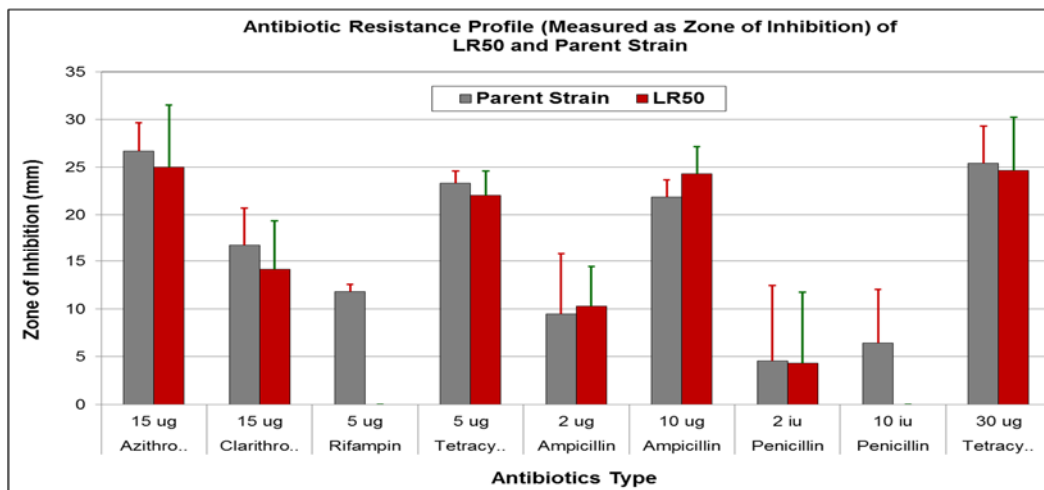


Figure 5. Antibiotic-resistant profiles of LR50 and PS for 9 antibiotics.

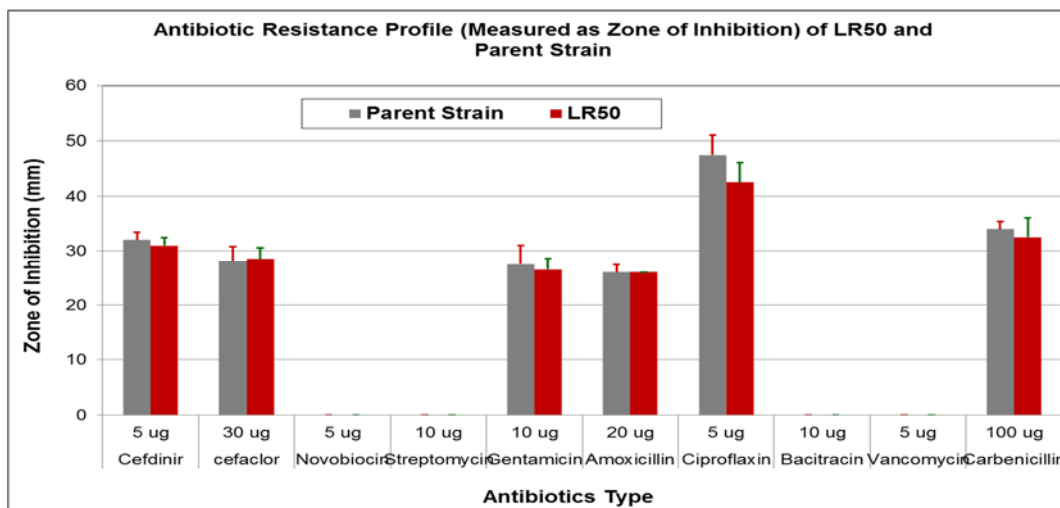


Figure 6. Antibiotic-resistant profiles of LR50 and PS for 10 antibiotics.

### 3.4 Genomic Sequencing

DNA was isolated from the PS and LR50 strains, and the genome sequences of both strains were compared. Certain single nucleotide polymorphisms (SNPs) are unique to LR50, including *acrB* and *mdtB* (multidrug efflux system proteins), subunit B (*yjfW*, inner membrane protein), and *yghB* (inner membrane protein). The fact that the multidrug efflux system protein was altered in the LR50 strain, but not in the PS, could be the likely cause of resistance because multidrug efflux pumps are transport proteins that eliminate toxins from within bacterial cells.

## 4. DISCUSSION

The growth and subculture of LR *E.coli* cells were continued for a 6 month duration in increasing concentrations of Lysol until the cells were able to grow in the presence of 50% of the RTU Lysol. This is consistent with findings in other studies. In one study (Hoff et al., 1986), *E. coli* was shown to have enhanced resistance to chlorine after repeated exposures. The phenotype of LR50 (i.e., LR *E.coli* cells) was confirmed by subculturing five times in TSB (in the absence of Lysol) and then growing cells in the presence of 50% RTU Lysol. Frozen glycerol stocks were prepared and the LR50 phenotype was confirmed by once again culturing cells in the presence of 50% Lysol. In another study (Moen, B. et al., 2012), *E.coli* cells were grown in the presence of quats and each generation of cells showed resistance to it.

Growth curves were performed with PS *E. coli* cells grown in TSB and 50% Lysol, LR50 cells grown in TSB and in 50% Lysol (Figures 1–3). The doubling times for PS and LR50 strains in TSB were about 60 min. The doubling time for LR50 in 50% Lysol was 65 min. As expected, PS *E. coli* cells did not grow in 50% Lysol.

The preliminary results showed differences in the protein levels of PS and LR50 strains, which suggested alterations in gene expression as a basis for LR50 growth in 50% Lysol (Figure 4). The LR strain grown in 50% Lysol was missing a polypeptide of ~130 kDa, which was present in the PS and LR strains grown in TSB. The LR50 strain grown in 50% Lysol had a polypeptide of ~100 kDa that was missing in both the PS and LR strains grown in TSB. This polypeptide could be the reason for the disinfectant resistance, but more studies need to be conducted to verify the identity of the polypeptide.

Nineteen antibiotics were tested against the PS and LR50 strains. The LR50 strain had no zone of inhibition around the 5 µg of rifampicin and the 10 IU of penicillin, whereas the PS had a zone of inhibition around these two antibiotics (Figures 5 and 6). Only the LR50 strain was observed to be resistant to 5 µg of rifampicin and 10 IU of penicillin. These data disagree with one study, which proposed that no correlation existed between antibiotic and disinfectant resistance (Beier et al., 2013).

DNA was isolated from the PS and LR50 strains, and the genome sequences of both strains were compared. The following SNPs were unique to LR50: *acrB* (multidrug efflux

system protein), *mdtB* (multidrug efflux system), and subunit B, *yjfW*, and *yghB* (inner membrane proteins).

The LR50 strain likely showed resistance because the multidrug efflux system protein altered in the LR50 strain and not in the PS. Multidrug efflux pumps are transport proteins that eliminate toxins from within bacterial cells. In one study, Sulavik et al. (2001) found that the multidrug resistance pumps contributed to the resistance of *E. coli* cells to antibiotics and different chemicals. Our hypothesis is that alteration in the multidrug efflux pump enabled the LR50 strain to grow in the presence of Lysol.

## **5. CONCLUSIONS**

In this study, bacterial cells were shown to acquire resistance to increasing concentrations of 50% RTU Lysol. This adaptive phenotype appeared to change in gene expression (i.e., specific proteins were gained and lost). The genome sequencing of the PS and resistant strains showed SNPs in the genome of the Lysol-resistant strain, corresponding to the multidrug efflux pumps. The efflux pumps may have cycled the disinfectants causing this resistance. Finally, this study addressed the very fundamental question of whether disinfectant strains could show altered antibiotics resistance. The results summarized in this report strongly support the idea that cells resistant to disinfectants also display altered resistance to antibiotics.

More studies need to be performed, especially studies addressing the adaptive potential of pathogenic strains, such as *Acinetobacter baumannii*, *Pseudomonas diminuta*, and *S. aureus*, against sublethal doses of disinfectants like Germ-X (a hand sanitizer) and quats. Further work on SNPs is expected to shed light on the specific gene or set of genes that can be considered for phenotype study.



## LITERATURE CITED

1. Beier, R.C.; Pool, T.L.; Brichta-Harhay, D.M.; Anderson, R.C.; Bischoff, K.M.; Hernandez, C.A.; Bono, J.L.; Arthur, T.M.; Nagaraja, T.G.; Crippen, T.L.; Sheffield, C.L.; Nisbet, D.J. Disinfectant and Antibiotic Susceptibility Profiles of *Escherichia coli* O157:H7 Strains from Cattle Carcasses, Feces, and Hides and Ground Beef from the United States. *J. Food Prot.* **2013**, *76* (1), 6–17.
2. Hoff, J.C.; Akin, E.W. Microbial Resistance to Disinfectants: Mechanisms and Significance. *Environmental Health Perspective* **1986**, *69*, 7–13.
3. McDonnell, G.; Russell, A.D. Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews* **1999**, *12* (1), 147–179.
4. Moen, B.; Rudi, K.; Bore, E.; Langsrud, S. Sub minimal Inhibitory Concentrations of the Disinfectant Benzalkonium Chloride Select for a Tolerant Subpopulation of *Escherichia coli* with Inheritable Characteristics. *Int. J. Mol. Sci.* **2012**, *13*, 4101–4123.
5. Sulavik, M.C.; Houseweart, C.; Cramer, C.; Jiwani, N.; Murgolo, N.; Greene, J.; DiDomencio, B.; Shaw, K.J.; Miller, G.H.; Hare, R.; Shimer, G. Antibiotic Susceptibility Profiles of *Escherichia coli* Strains Lacking Multidrug Efflux Pump Genes. *Antimicrobial Agents and Chemotherapy* **2001**, *45* (4), 1126–1136.

Blank

## ACRONYMS AND ABBREVIATIONS

ATCC	American Type Culture Collection
LR	Lysol resistant
LR50	Lysol resistant in 50% Lysol
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
PS	parent strain
quats	quaternary ammonium compounds
RTU	ready-to-use
SDS-PAGE	sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
TSB	Tryptic soy broth

Blank

## **DISTRIBUTION LIST**

Each of the following individuals or organizations were provided with one Adobe portable distribution format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical  
Biological Center (ECBC)  
RDCB-DRB-D

Attn: Smith, L.  
Rastogi, V.  
Wallace, L.  
Rosenzweig, N.  
Broomall, S.

G-3 History Office  
U.S. Army RDECOM  
Attn: Smart, J.

ECBC Technical Library  
RDCB-DRB-BL  
Attn: Stein, J.  
Foppiano, S.

Defense Threat Reduction Agency  
DTRA/RD-CBD T  
Attn: Pate, J.  
(J9-CBS), MSC 6201  
Attn: Moore, E.

Office of the Chief Counsel  
AMSRD-CC  
Attn: Upchurch, V.

Department of Homeland Security  
DHS ORD CSAC  
Attn: Famini, G

Defense Technical Information Center  
Attn: DTIC OA

ECBC Rock Island  
RDCB-DES  
Attn: Lee, K.

